PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/569, 33/50 — A1

(11) International Publication Number: **WO 99/66331**

(43) International Publication Date: 23 December 1999 (23.12.99)

(21) International Application Number: PCT/GB99/01929

(22) International Filing Date: 17 June 1999 (17.06.99)

(30) Priority Data:

9813112.1	17 June 1998 (17.06.98)	GB
9813215.2	18 June 1998 (18.06.98)	GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

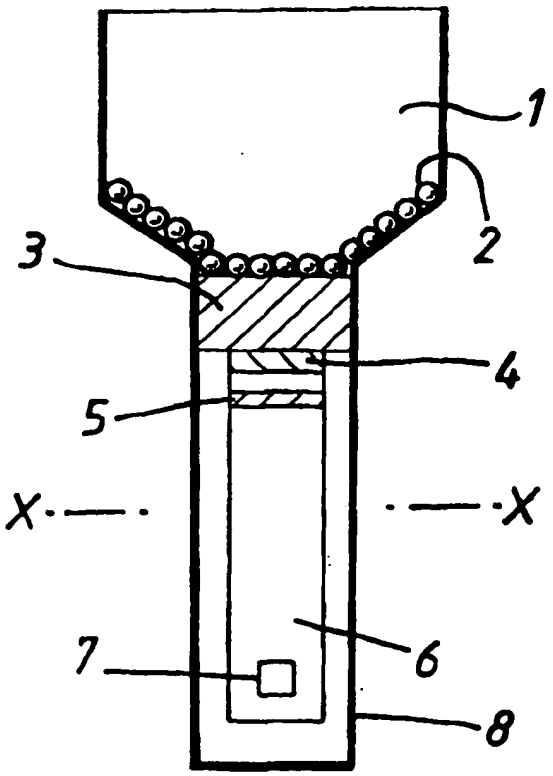
Published

With international search report.

(54) Title: METHOD AND KIT FOR THE DETECTION OF MALE INFERTILITY

(57) Abstract

A method and kit for (i) separating motile spermatozoa from non–motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa, comprising depositing a sample of spermatozoa onto a porous layer of thickness 25–2500 μm and allowing any motile spermatozoa contained in said sample to migrate through the porous layer to a detection zone, where said motile spermatozoa are detected.



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METHOD AND KIT FOR THE DETECTION OF MALE INFERTILITY

The present invention relates to a method and kit for the detection of motile spermatozoa in a sample, such a method and kit being useful in a number of applications, including the detection of male infertility.

BACKGROUND OF THE INVENTION

It has been estimated that approximately 14-16% of all couples attempting to get pregnant have difficulty in conceiving and are defined by fertility therapists as infertile. 40% of these cases result from male factors. In a substantial proportion of these, treatment is available to ameliorate or relieve the condition which leads to infertility.

Other conditions also exist in which it is desirable to test for the presence or otherwise of viable spermatozoa in a sample. For example, vasectomies are now frequently carried out as a method of contraception, but it is necessary to verify the effectiveness of a vasectomy by confirming that ejaculate is free of viable spermatozoa for a period of time after the operation.

A number of methods exist for assessing the motility and number of spermatozoa in a sample. One such method is microscopic analysis, which is typically carried out in a hospital or commercial laboratory. More recently, however, a number of proposals have been made for test kits which are intended to simplify the detection of spermatozoa, and which may therefore be useful in the diagnosis of male infertility. For example, WO97/40386 discloses a kit which is based on the detection of the 34kD human epididymal spermatozoa protein (P34H). This protein is thought to be involved in spermatozoa-zona pellucida interaction. The test kit disclosed in WO97/40386 uses an antibody raised against P34H or a related antigen, and a reagent for detecting antibody binding to P34H. As disclosed in WO97/40386, spermatozoa in a test sample are washed three times by centrifugation in Dulbecco-phosphate buffered saline. The samples are then heat denatured at 95°C, centrifuged at 14000g, and the supernatants are then used for analysis.

EP-A-0387873 also discloses a kit for the evaluation of male fertility. This kit uses solid beads to which is bound an antibody specific to an antigenic site on the human spermatozoon acrosome. Such beads are mixed with a test sample, and incubated for a period of 10 to 30 minutes. The test beads are then separated from the suspension, washed and subjected to

measurement of the number of spermatozoa bound to the solid beads, preferably by examination with the aid of a microscope.

A kit for the detection of spermatozoa in a sample is also disclosed in WO95/29188. In this case, the test is based on antibodies to an antigen such as the SP-10 antigen of human spermatozoa.

A significant disadvantage of the test kits disclosed in the prior art mentioned above is that they do not distinguish between motile and non-motile spermatozoa. In the detection of male infertility, the ability to assess the numbers of motile spermatozoa is the most predictive indicator of male infertility. Moreover, many of the prior art test kits involve procedures, such as centrifugation or microscopic examination, which do not lend themselves to home use.

SUMMARY OF THE INVENTION

The present invention provides a method and a kit relating thereto for (i) separating and/or (ii) detecting motile spermatozoa, which can be used in the home to indicate the number of motile spermatozoa in a sample. The kit can therefore be used as a diagnostic tool for the assessment of male fertility.

Accordingly, the present invention provides a method of (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa, comprising depositing a sample of spermatozoa onto a porous layer of thickness 25-2500 μm and allowing any motile spermatozoa contained in said sample to migrate through the porous layer to a detection zone, where said motile spermatozoa are detected.

Preferably, the porous layer has a thickness of 50-1000 μm, more preferably 80-500 μm.

The porous layer preferably has a pore size of 5-100 μm, more preferably 8-60 μm, most preferably 10-40 μm. Preferably, the porous layer is fibrous and can be made of glass wool or polypropylene.

The pore size of the porous layer at the site of sample deposition can differ from the pore size of the porous layer at the detection zone. Preferably, the pore size at the site of sample deposition is smaller than the pore size at the detection zone. Furthermore, the hydrophobicity

of the porous layer at the site of sample deposition can be greater than the hydrophobicity of the porous layer at the detection zone.

Decreasing the pore size and/or increasing the hydrophobicity of the porous layer towards the site of sample deposition means that capillary movement of any liquid associated with the spermatozoa is reduced, thereby ensuring that only motile spermatozoa reach the detection zone and that non-motile spermatozoa or spermatozoa of reduced motility are retarded through the lack of a solvent front with which they can migrate. Decreasing the pore size and/or increasing the hydrophobicity of the porous layer towards the site of sample deposition is particularly preferred if the spermatozoa have not already been subjected to a filter to separate motile spermatozoa from non-motile/reduced motility spermatozoa.

The detection zone preferably comprises a reagent or a combination of reagents which is/are directly or indirectly capable of generating a visual signal on interaction with spermatozoa. The reagent or combination of reagents is preferably immobilised at the detection zone, so as to provide a more readily visible signal at the detection zone. Methods of immobilising reagents in porous materials are well known in the art. The reagent or combination of reagents can include antibodies that detect an antigen that is present on spermatozoa and are capable of binding spermatozoa. The spermatozoa, when immobilised by these antibodies, can be visually detected using a visually detectable reagent specific to spermatozoa.

The present invention also provides a method as described above, wherein a spermatozoa chemoattractant is located away from the site of sample deposition towards the detection zone. Preferably, the spermatozoa chemoattractant is located at or beyond the detection zone. An example of a suitable spermatozoa chemoattractant is follicular fluid (Villanueva-Díaz C. *et al.*, 1995).

The porous layer can be supported on either or both sides of the layer, preferably by means of a plastic backing.

The present invention further provides a method as described above, wherein a pick-up zone is located between the site of sample deposition and the detection zone, said pick-up zone comprising a reagent or combination of reagents which is/are capable of binding to spermatozoa and being transported therewith through the porous layer to the detection zone.

Preferably, the reagent or combination of reagents of the pick-up zone include antibodies that detect an antigen that is present on spermatozoa. More preferably, these antibodies are detectably labelled. Most preferably, these antibodies are detectably labelled with gold particles. The antibodies in the detection zone that detect an antigen that is present on spermatozoa preferably recognise a different spermatozoa antigen compared to the antibodies of the pick-up zone.

According to a yet a further aspect of the present invention, there is provided a method as described above, wherein the detection zone comprises, or further comprises, a spermatozoa acrosome-lysing reagent and a means for detecting pH change. Preferably, the spermatozoa acrosome-lysing reagent is a lysis buffer. More preferably, the lysis buffer comprises Proteinase K or the calcium ionophore A23187.

The detection means is preferably a pH sensitive probe or a pH indicator reagent capable of visually detecting a pH change. The pH indicator reagent can be bromocresol purple.

The present invention also provides a kit for (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa, comprising:

(a) a receptacle for receiving a spermatozoa-containing sample;

(b) a porous layer as defined above, optionally housed in a housing means; and

(c) a conduit communicating with the receptacle and sample deposition site of the porous layer, said conduit providing a path along which spermatozoa can pass from the receptacle to the porous layer .

Preferably, the conduit comprises a filter which is capable of separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility, thereby allowing motile spermatozoa to pass through the filter from the receptacle onto the site of deposition. The filter is preferably glass wool, polypropylene, gel or foam.

The receptacle of the kit described above preferably contains a spermatozoa-liquifying reagent.

The present invention yet further provides a kit for (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii)

detecting said motile spermatozoa substantially as hereinafter described with reference to, and as shown in, the accompanying drawings.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described, by way of example, with reference to the following Figures, wherein:

Figure 1 shows a cross-sectional view of a kit of one embodiment of the present invention, which illustrates a porous layer (6) with a pick-up zone (5) and detection zone (7) and a site of deposition (4) in communication via a filter-conduit (3) to a sample-receiving receptacle (1), optionally containing a spermatozoa liquifying reagent (2). The apparatus is housed in a housing means (8); and

Figure 2 shows a cross-sectional, schematic view of the porous layer of Fig. 1 across point X-X showing (schematically) a plastic backing (9) supporting a porous layer (6).

Separation of the detection zone from the sample-receiving receptacle (with an intervening separating filter-conduit to separate motile spermatozoa from non-motile/reduced motility spermatozoa or a porous layer pore structure which acts as said separating filter-conduit) has the advantage that non-motile/reduced motility spermatozoa do not reach the reagent or reagents in the detection zone, and therefore do not generate a visual signal. Moreover, movement of the spermatozoa along the conduit and porous layer can be used to separate spermatozoa from other seminal plasma proteins, which might otherwise interfere with the detection method used in the detection zone.

Seminal plasma proteins can interfere with many aspects of spermatozoa function. Some seminal plasma proteins are known to bind to spermatozoa. There is considerable evidence to suggest that they work as decapacitation factors (i.e. prevent capacitation – the ability of spermatozoa to fertilize an oocyte), at least in animals. Such proteins (and other molecules) are removed from spermatozoa when they are in the female genital tract. Examples of such proteins include spermadhesins (AWN 1 and 2; Calvete *et al.*, 1993, 1994, 1997). In mice an, as yet, unidentified factor in seminal plasma which stimulates calcium ATPase (Adeoya-Osiguwa and Fraser, 1996) and thus acts as a decapacitation factor has been described. In humans, seminal plasma does adversely effect spermatozoa capacitation but, as yet, no clearly defined molecules have been identified. A spermatozoa motility inhibitor has been identified

in humans (Robert and Gagnon, 1995) but the effect on spermatozoa capacitation is unknown. In addition, an acrosome reaction-preventing substance has been detected in human seminal plasma (ARIG; Drisdel *et al.*, 1995).

As used herein, the term "layer" is not limited to flat sheet-like strips, but may also comprise, for example, thin tube-like or channel-like structures. For example, the layer may comprise a porous open-cell foam, the cells of the foam containing a medium which is physiologically acceptable to spermatozoa. Alternatively, the layer may comprise a porous gel structure, the gel being sufficiently solid to maintain its physical structure, while still being permeable to spermatozoa. The layer may, however, comprise a porous fibrous material such as glass wool, polypropylene or some such similar substance known in the art (see, for example, Mortimer, 1994).

The layer may be supported by, for example, a backing on either side of the layer (or completely enclosing said layer). For economy of manufacture, the layer support, tube or channel is preferably made of plastics material which is non-toxic to spermatozoa, such as Falcon®.

If the layer comprises a tube or channel containing a liquid or gel medium, such liquid or gel medium is preferably buffered at a physiological pH, for example, between a range of pH 6.5-7.6. Examples of such useful buffers would include Hepes buffered media. Media which could be appropriately buffered include Biggers, Whitten & Whittington (BWW; Biggers *et al.*, 1971), Earle's balanced salts (EBS; Purdy *et al.*, 1982), Hams F10 (Lopata *et al.*, 1980; Dandekar and Quigley, 1984), Human Tubal Fluid (Quinn *et al.*, 1985), Menezo's B2 (Ménézo, 1976; Menezo *et al.*, 1984) and Synthetic Tubal Fluid (Mortimer, 1986). Protein (human serum albumin) would preferably be added to the media at a concentration of 1 g in 100 ml. The osmolarity of the solution would preferably be between 270-300 mOsm.

Gel compositions are particularly preferred, as they facilitate handling of the test kit. Suitable gelling agents include polysaccharides such as hyaluronic acid (usually added as sodium hyaluronate (molecular weight >400,000 daltons) at a concentration of 10 mg/ml of culture medium; available commercially as Healonid®, Pharmacia and Upjohn, Uppsala, Sweden (Perry *et al.*, 1996)), Viscoat® (sodium chrondroitin (mean molecular weight approximately 22,500 daltons) and sodium hyaluronate) available commercially from Alcon Laboratories (UK) Ltd. and Percoll®, Pharmacia, Uppsala, Sweden (Mortimer 1994).

However, the layer most preferably comprises a fibrous composition such as glass wool or polypropylene. The use of a glass wool column has been reported to remove most of the debris as well as agglutinated and dead (i.e. non-motile) spermatozoa from semen samples (Paulson and Polakoski, 1977 and Rhemrev *et al.*, 1979 as reported in Mortimer 1994).

The detection zone of the porous layer may be impregnated with the required detection reagent or reagents. In some embodiments, the reagent or reagents in the detection zone are immobilized by being bound to the porous layer or bound to a support over which is laid the (thin) porous layer. In other, less preferred. embodiments, the reagent or reagents may be in solution in a gel.

Detection of spermatozoa in the detection zone can be by any of a wide varicty of means. In one embodiment, detection is based on the fact that the pH inside the acrosome of a spermatozoon differs from the pH of the surrounding medium (Brook *et al.*, 1996; Cross and Razy-Faulkner, 1997). In this embodiment of the present invention, the detection zone contains a pH indicator reagent, such as bromocresol purple, together with a reagent, such as Proteinase K or the calcium ionophore A23187 (Perry *et al.*, 1995, 1996, 1997ab), which causes the acrosomes to open, thereby causing a pH change detectable by the pH indicator reagent. A preferred reagent for lysing acrosomes is a lysis buffer comprising 2% SDS, 100 µg/ml Proteinase K in 10 mM Tris-HCl and 0.1 M EDTA, in which the spermatozoa are incubated for approximately 1 h. The lysis composition can include a pH indicator reagent such as bromocresol purple (Sigma, UK) which changes colour from purple to green once the spermatozoa acrosomes are lysed (controls use no lysis buffer or can substitute nuclear lysis buffer in place of the acrosome lysis buffer – no change of colour of the pH indicator reagent is observed in such cases).

More usually, the detection zone will contain a reagent which binds to intact spermatozoa or to one or more of the components thereof, and this binding reaction is used to generate a visual signal.

Suitable binding reagents include monoclonal or polyclonal antibodies, which can, for example, be tagged with an enzyme or substrate. Examples of such antibodies include antibodies against the putative membrane progesterone receptor identified by Meyer *et al.*, 1996 or antibodies against zona receptor kinase (Burks *et al.*, 1995). Alternatively, instead of using antibodies to known surface antigens, one could identify suitable antigenic candidates by

examining which molecules on the spermatozoa bound to the zona pellucida. Spermatozoa binding to the zona pellucida is a critical step in the spermatozoa-oocyte fertilisation process. Solubilised (native) human zona pellucida (or recombinant human or animal zona proteins (or glycoproteins)) could be used to identify molecules on spermatozoa which cross-react with the zona material. The spermatozoa-zona pellucida interaction is well-documented (see, for example, Chapman and Barratt, 1996, 1997; Chapman *et al.*, 1998; and Whitmarsh *et al.*, 1996). The techniques used to identify the complementary molecules on the spermatozoa surface could involve the use of cross-linker reagents, labelling using tags (for example, biotin, histidine or strepavidin), cellulose beads, fluorescence or immunofluorescence (with antigen labelling or using chlortetracycline (Perry *et al.*, 1995, 1997ab)). The binding may be detected on live or prepared spermatozoa (for example, spermatozoa prepared on one or two-dimensional gels). Useful proteins would be identified by using protein sequencing. Antibodies would then be made either by producing recombinant protein in a prokaryotic system, for example, and/or designing immunogenic peptides.

Instead of using antibodies as probes for the spermatozoa, other techniques could be utilised such as the use of nucleic acid material, for example oligonucleotides (see, for example, the SELEX procedure – Gold *et al.*, 1997; Cho *et al.*, 1997, Eaton *et al.*, 1997) or RNA probes. Alternatively, receptors for spermatozoa-specific molecules (e.g. binding sites for steroids) could be used - examples include a novel progesterone receptor site (Meyer *et al.*, 1998, Luconi *et al.*, 1998) or a novel prostaglandin E receptor (Schaefer *et al.*, 1998).

Methods of visually detecting molecules in association with or bound to spermatozoa include tagging/labelling such molecules (for example, enzyme/substrate interactions), reactive oxygen species generation, or complementary tagging (for example, strepavidin as used in the Strep tags™ purification technique, Biometra, USA).

In one embodiment of the invention, the detection zone will comprise antibodies that detect an antigen present on spermatozoa which are capable of binding and immobilising spermatozoa. A sample of spermatozoa will be deposited on the site of deposition on the porous layer, preferably having first passed from a sample-receiving receptacle and through a filter-conduit. The motile spermatozoa of the sample will then be able to travel through the porous layer to the detection zone, where they will be detected.

The porous layer will be of such a material and of such a thickness that it will act to (further) separate motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility. Thus, if the spermatozoa have not already been subjected to a filtration/separation step to remove debris, other seminal proteins and non-motile and/or spermatozoa with reduced motility prior to deposition onto the porous layer, then the porous layer of the present invention can provide a suitable medium in which such filtration/separation can occur.

It should be noted that the cause of non-motility or reduced motility in spermatozoa is not only related to the absence of a suitably functioning flagellum. Abnormal spermatozoa morphology can also cause or contribute to impairment of spermatozoa motility. For example, an abnormally large-headed or multiple-headed spermatozoa would not be able to negotiate the pores of the porous material (which would, in practice, be smaller than the size of the abnormal spermatozoa head) in order to reach the detection zone.

The pore size of the porous layer is an important factor in the operation of the present invention. The pores of the porous layer must be large enough to allow the head (the widest section) of normal spermatozoa to pass though, but not so large as to allow spermatozoa to passively 'fall' through. To further retard the passive movement of spermatozoa through the pores of the porous layer, the pores should preferably be arranged so that a direct (essentially straight) passage through the layer is not possible (i.e. the pores should be arranged in an irregular manner such that they must be "negotiated" by the motile spermatozoa – the motile spermatozoa being the only seminal entities capable of "weaving" through the pore structures). Furthermore, the pore structure should be such as to reduce the possibility of capillary action, which would otherwise aid the passive movement of non-motile spermatozoa and/or spermatozoa with reduced motility via association with the solvent front. Such a reduction in or elimination of capillary action may be effected by differential pore size throughout the porous layer. Thus, having smaller pores towards the site of spermatozoa deposition on the porous layer and having ever increasing pore sizes going towards the detection zone, the capillary action of any liquid present would be hindered the further along the porous layer any solvent front were to travel as the larger pore size would act against capillary action. Moreover, the motile spermatozoa would be "forced" to travel in the direction of the detection zone, as the increasing pore size would aid their progress. Any non-motile spermatozoa and/or spermatozoa with reduced motility would be retained in the small pore size area towards the site of deposition. Capillary action could also be reduced or eliminated by increasing the

hydrophobicity of the pores of the porous layer towards the site of deposition. This would repel the solvent front and hinder its advancement through the porous layer. Increasing the hydrophobicity in an area of the porous layer could be achieved in a number of different ways, which could include chemically treating the area or manufacturing the area from a hydrophobic material.

However, if the spermatozoa have already been separated according to motility, then the presence of capillary action through the pore structure is optional. It will be therefore be appreciated that the important factor of the present invention is that only motile spermatozoa are able to reach the detection zone of the porous layer. If a mixture of motile and non-motile/reduced motility spermatozoa is subjected to a method according to the present invention (without prior filtration to separate motile spermatozoa from non-motile spermatozoa or spermatozoa with reduced motility), then the pore structure/composition will preferably be such as to retard capillary action so as to avoid transporting non-motile/reduced motility spermatozoa to the detection zone via the solvent front. If, however, only motile spermatozoa are moving towards the detection zone, then the pore structure/composition may or may not retard capillary action and the motile spermatozoa may or may not reach the detection zone by travelling with the solvent front (if any exists).

The thickness of the porous layer is also an important factor in the operation of the present invention. Although plugs and columns of porous material have been used to separate motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility, it has advantageously been found that the same function can be performed when the porous material is formed in a thin layer. It has been found that a thickness of the porous layer between 25-2500 μm can not only separate motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility (if required, i.e. if no prior filtration/separation step is included), but is also able to ensure the survival of the motile spermatozoa as they pass through the thin porous layer.

The survival of motile spermatozoa in the method of the present invention is an important factor. Spermatozoa are known to desiccate, become non-motile and subsequently die when exposed to air. By reducing the thickness of the porous layer, the relative exposure of the spermatozoa to air is increased and, as a consequence, it was expected that the desiccation, non-motility rate and death rate of the spermatozoa would also increase. Surprisingly,

however, we have shown survival rates for motile spermatozoa of up to two hours when such spermatozoa pass through a thin porous layer of only between 25-2500 μm in thickness.

The reduced thickness of the porous layer also means that fewer motile spermatozoa are required to produce a positive signal capable of visual detection by the naked eye, thus making the method more sensitive. Having a detection reagent located within, for instance, the thick bulk of a glass wool plug or column would suffer from the problem that an increased amount of such a reagent would be required in order to visualise any positive signal through the thickness of the surrounding material. This in turn would require that more motile spermatozoa would have to reach the detection zone in order to generate a strong enough positive signal through the activation of the increased amount of detection reagent that would be visible to the naked eye through the thickness of the layer, thereby rendering the method less sensitive.

Clearly, such a discovery allows spermatozoa motility test kits to be manufactured on a smaller scale than before and therefore more readily available and convenient for use at home, in the GP surgery or in a clinic/hospital setting.

Preferably, the porous layer will further comprise a pick-up zone between the site of deposition and the detection zone, through which the motile spermatozoa must pass to get to the detection zone. The pick-up zone will preferably comprise further antibodies that detect an antigen present on spermatozoa which are visually detectable, preferably labelled. The motile spermatozoa therefore pass from the site of deposition and into the pick-up zone, where the spermatozoa bind to the visually detectable antibodies that detect an antigen present on spermatozoa and then continue to travel past the pick-up zone towards the detection zone "carrying" the bound antibodies. Once at the detection zone, the antibodies located in the zone that detect an antigen present on spermatozoa immobilise the spermatozoa (still in association with the other bound antibodies) and the visually detectable antibodies "carried" from the pick-up zone to the detection zone will be capable of direct or indirect detection. Methods of direct detection include the visualisation of a label attached to the pick-up zone antibodies. Such labels could include gold particles - visualised as pink at a particle concentration of 20 μl at OD_1 (= 1.8×10^9 particles (1 ml at OD_1 is equivalent to 9×10^{10} particles)) or more. Thus, the gold particle-labelled antibodies of the pick-up zone would be visualised as a pink band that would migrate from the pick-up zone to the detection zone in the presence of motile

spermatozoa. Alternatively, the detection antibodies could be anti-gold particle antibodies or, at least, antibodies specific for the antibodies of the pick-up zone.

Of course, other reagents capable of performing the above-noted functions attributed to antibodies would also be useful in the operation of the present invention.

In yet a further embodiment, the motile spermatozoa may be aided in their progress from the site of deposition to the detection zone by way of a spermatozoa chemoattractant located in the area of the detection zone, preferably in the area behind the detection zone relative to the site of deposition. Such chemoattractants include follicular fluid, which attracts the spermatozoa to the chemical via a chemotactic response in the spermatozoa (Villanueva-Díaz C. *et al.*, 1995).

EXAMPLES

Example 1: Viability assessment of spermatozoa in thin fibrous glass wool strips

The maintenance of viability of spermatozoa in a thin (300 µm, pore size 20 µm) glass wool membrane was examined to assess its value to isolate motile spermatozoa for subsequent detection by antibody/enzyme based assays. One end of a glass wool membrane strip (approximately 2 cm in length) was dipped into a well of liquefied semen for 30 sec. The membrane was then placed on a slide and overlain by a coverslip. Under phase contrast microscopy, motile spermatozoa were observed within the matrix at the point of sample application. The slide was then incubated at room temperature in a humid chamber and observed at 30 min intervals over 2 hours. At the end of the 2-hour period, motile spermatozoa were still evident within the thin fibrous glass wool strip.

Example 2: Assessment of a separation technique using a thin 10 µm pore size polypropylene mesh filter

10 µm pore size (approximately 120 µm thick) polypropylene mesh centrifuge tube filters (VectaSpin®,Whatman Inc., USA) were soaked with medium (Earles solution) by the following method: 1 ml of medium was placed in the centrifugation tube and the filter insert was added. 0.5 ml of medium was placed in the insert and the assembly was spun at 500 *g* for 10 min until the medium had spun through the filter completely to fill the bottom tube and wet the membrane. The residual medium was then carefully removed and replaced with 0.5 ml of liquefied semen. Following an incubation of 10 min the filtrate medium was analysed by computer assisted semen analysis (CASA) and a dead count was performed.

A comparative separation technique was also performed using a commercially available glass wool plug optimised for separation of motile spermatozoa (Cook® Sperm Filter, Order No. J-CSF-010000; Cook IVF, UK). This thick (3 mm) glass wool plug was used as per the manufacturer's instructions with an incubation time of 10 min.

Results:

	Semen Sample	10 µm Mesh Filtrate	Cook® Filtrate
Count	**86 x 10^6 /ml**	**3.3x10^6/ ml**	**20.3 x 10^6 /ml**
Motility	**70%**	**100%**	**84%**
Rapid	**53%**	**50%**	**50%**
Moderate	**8%**	**17%**	**22%**
Slow	**9%**	**13%**	**13%**
Stationary	**30%**	**0%**	**16%**

This experiment demonstrates the effectiveness of separating motile spermatozoa using a thin fibrous matrix.

Example 3: Antibody tagging of the CD59 antigen post micro mesh filtration

The antibody BRIC 229 detects the complement regulatory protein CD59. This antigen is broadly distributed on human cells including spermatozoa. Rooney *et al.* (1992) demonstrated that CD59 is present on the surface of spermatozoa and is present on average in 95% (range 87-98%) of spermatozoa obtained by swim-up separation.

The experiment described below demonstrates that the CD59 antigen remains intact after passing through a thin fibrous filter.

400 µl of the 10 µm pore size mesh separation filtrate (obtained as in Example 3) was placed in a conical 10 ml tube and 100 µl of BRIC 229 antibody was added. The sample was incubated for 1 hour with frequent agitation.

Following incubation the cells were washed 3x by centrifugation at 600 *g* with PBS. The cells were finally resuspended in 400 µl of PBS. Smears were prepared on glass slides, air dried for 45 min and fixed in ice cold acetone (10 min). A second antibody (rabbit anti-mouse-peroxidase conjugate) P260 diluted to 0.025 in PBS was added to the smear. The slide was further incubated for 40 min at room temperature in a humid chamber. The slides were developed with diaminobenzidine (DAB; Sigma, UK) and the reaction stopped by running

under tap water. The spermatozoa were stained brown indicating that the CD59 antigen remains intact after passage through the thin fibrous filter system.

Example 4: Detection of pH change in lysed versus non-lysed spermatozoa

We wanted to determine whether we could detect a pH change when spermatozoa were present in a sample and subsequently lysed. The spermatozoa samples were prepared by the swim-up technique as described by Mortimer (1994). Subsequently, the spermatozoa were lysed and a colour change, indicative of pH change, was visually observed. The spermatozoa were lysed using lysis buffer (2% SDS, 100 µg/ml Proteinase K in 10 mM Tris-HCl and 0.1 M EDTA and incubated for 1 hour). Bromocresol purple (Sigma, UK) was used as the colour indicator and the colour changed from purple to green/yellow. No change in colour was observed in the controls (no lysis buffer and/or nuclear lysis buffer –Promega, UK).

It will, of course, be understood that the present invention has been described above by way of example only, and that modifications of detail can be made within the scope and spirit of the invention as defined in the appended claims.

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Claims

1. A method of (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa, comprising depositing a sample of spermatozoa onto a porous layer of thickness 25-2500 μm and allowing any motile spermatozoa contained in said sample to migrate through the porous layer to a detection zone, where said motile spermatozoa are detected.

2. The method of claim 1, wherein the porous layer has a thickness of 50-1000 μm, preferably 80-500 μm.

3. The method of claim 1 or claim 2, wherein the porous layer has a pore size of 5-100 μm, preferably 8-60 μm, more preferably 10-40 μm.

4. The method of any one of claims 1 to 3, wherein the porous layer is fibrous.

5. The method of claim 4, wherein the fibrous layer is made of glass wool or polypropylene.

6. The method of any one of claims 1 to 5, wherein the pore size of the porous layer at the site of sample deposition differs from the pore size of the porous layer at the detection zone.

7. The method of claim 6, wherein the pore size at the site of sample deposition is smaller than the pore size at the detection zone.

8. The method of any one of claims 1 to 7, wherein the hydrophobicity of the porous layer at the site of sample deposition is greater than the hydrophobicity of the porous layer at the detection zone.

9. The method of any one of claims 1 to 8, wherein the detection zone comprises a reagent or a combination of reagents which is/are directly or indirectly capable of generating a visual signal on interaction with spermatozoa.

10. The method of claim 9, wherein the reagent or combination of reagents include antibodies that detect an antigen present on spermatozoa and are capable of binding spermatozoa.

11. The method of claim 10, wherein the spermatozoa, when immobilised by the antibodies that detect an antigen present on spermatozoa, can be visually detected using a visually detectable reagent specific to spermatozoa.

12. The method of any one of claims 1 to 11, wherein a spermatozoa chemoattractant is located away from the site of sample deposition towards the detection zone.

13. The method of claim 12, wherein the spermatozoa chemoattractant is located at or beyond the detection zone.

14. The method of any one of claims 1 to 13, wherein the porous layer is supported on either or both sides of the layer.

15. The method of claim 14, wherein the support is plastic backing.

16. The method of any one of claims 1 to 15, wherein a pick-up zone is located between the site of sample deposition and the detection zone, said pick-up zone comprising a reagent or combination of reagents which is/are capable of binding to spermatozoa and being transported therewith through the porous layer to the detection zone.

17. The method of claim 16, wherein the reagent or combination of reagents include antibodies that detect an antigen present on spermatozoa.

18. The method of claim 17, wherein the antibodies that detect an antigen present on spermatozoa are detectably labelled.

19. The method of claim 18, wherein the antibodies that detect an antigen present on spermatozoa are detectably labelled with gold particles.

20. The method of claim 19, wherein the antibodies located in the detection zone that detect an antigen present on spermatozoa recognise a different spermatozoa antigen compared to the antibodies located in the pick-up zone that detect an antigen present on spermatozoa.

21. The method of any one of claims 1 to 20, wherein the detection zone comprises, or further comprises, a spermatozoa acrosome-lysing reagent and a means for detecting pH change.

22. The method of claim 21, wherein the spermatozoa acrosome-lysing reagent is a lysis buffer.

23. The method of claim 22, wherein the lysis buffer comprises Proteinase K or the calcium ionophore A23187.

24. The method of any one of claims 21 to 23, wherein the detection means is a pH sensitive probe or a pH indicator reagent capable of visually detecting a pH change.

25. The method of claim 24, wherein the pH indicator reagent is bromocresol purple.

26. A kit for (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa, comprising:

(a) a receptacle for receiving a spermatozoa-containing sample;

(b) a porous layer as defined in any one of the preceding claims, optionally housed in a non-porous housing means; and

(c) a conduit communicating with the receptacle and sample deposition site of the porous layer, said conduit providing a path along which spermatozoa can pass from the receptacle to the porous layer .

27. The kit according to claim 26, wherein the conduit comprises a filter which is capable of separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility, thereby allowing motile spermatozoa to pass through the filter from the receptacle onto the site of deposition.

28. The kit according to claim 27, wherein the filter is glass wool, polypropylene, gel or foam.

29. The kit according to any one of claims 26 to 28, wherein the receptacle contains a spermatozoa-liquifying reagent.

30. A kit for (i) separating motile spermatozoa from non-motile spermatozoa and/or spermatozoa with reduced motility in a sample and/or (ii) detecting motile spermatozoa substantially as hereinbefore described with reference to, and as shown in, the accompanying drawings.

Fig. 1

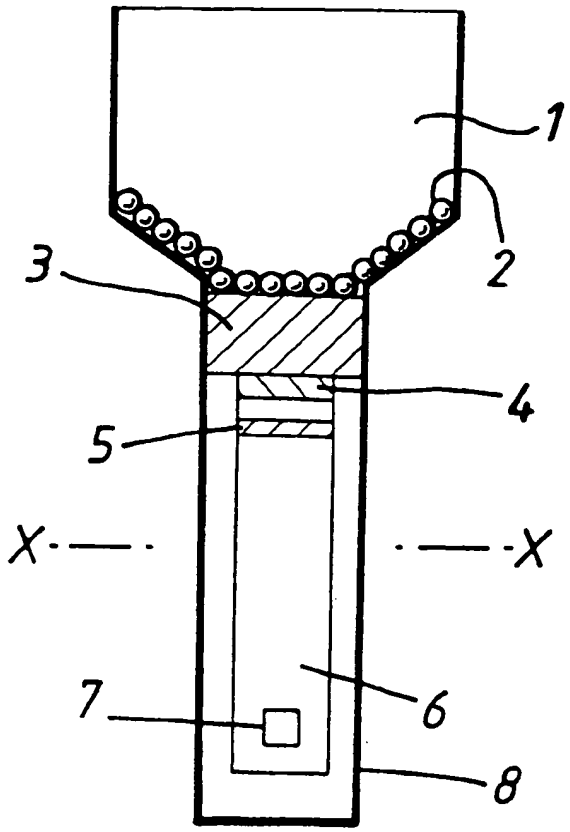
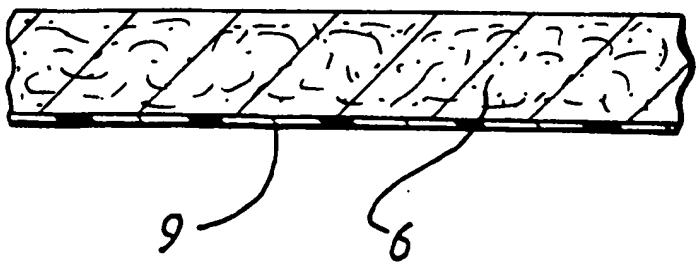


Fig. 2



INTERNATIONAL SEARCH REPORT

Inte tional Application No
PC /GB 99/01929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 13225 A (BETH ISRAEL HOSPITAL) 9 May 1996 (1996-05-09) claims 1,4,12,13 page 3, line 22 - page 4, line 2 page 6, line 9 - page 7, line 5 ---	1-30
X	US 5 575 914 A (JEYENDRAN RAJASINGAM S) 19 November 1996 (1996-11-19) claims column 3, line 30 - line 48 column 4, line 18 - line 42 ---	1-30
X	EP 0 302 765 A (SOEKAMI PHARMACEUTICAL RESEARC) 8 February 1989 (1989-02-08) claims 1,6,14,19 column 2, line 14 - line 17 column 3, line 13 - line 16 --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 September 1999	17/09/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inte tional Application No
PC1/GB 99/01929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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